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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 4 September 1995 in connection with Application No. PN 5172 for a patent by THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH filed on 4 September 1995.

I further certify that the annexed documents are not, as yet, open to public inspection.

WITNESS my hand this Twenty-seventh day of February 1996

DAVID DANIEL CLARKE
ASSISTANT DIRECTOR PATENT SERVICES

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THE WALTER AND ELIZA HALL
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RESEARCH

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PROVISIONAL SPECIFICATION
for the invention entitled:

"IMMUNOINTERACTIVE AND IMMUNOTHERAPEUTIC MOLECULES"

The invention is described in the following statement:

IMMUNOINTERACTIVE AND IMMUNOTHERAPEUTIC MOLECULES

The present invention relates generally to molecules such as peptides, polypeptides and proteins which interact immunologically with antibodies or T-cells in subjects having pre-clinical or clinical Insulin-Dependent Diabetes Mellitus (IDDM). These molecules are preferentially immunointeractive to T-cells in subjects having preclinical or clinical IDDM and are useful in the development of diagnostic, therapeutic and prophylactic agents for IDDM.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

IDDM results from the destruction of the insulin-secreting β - cells, probably mediated by T cells that recognise β-cell autoantigens. A major antigen implicated in T-cell mediated β-cell destruction characteristic of IDDM is glutamic acid decarboxylase (GAD), which occurs in two major isoforms, GAD 65 and GAD 67. These two isoforms have 65% similarity at the amino acid sequence level. Subjects with IDDM or at high-risk of the disease show autoantibody and autoreactive T-cell responses to GAD and/or insulin. In NOD mice, an animal model of spontaneous IDDM, GAD is a dominant and early target antigen (Tisch et al (1993) Nature 366:p72-75).

Identification of the immunodominant epitope(s) of pathogenic autoantigens (HLA molecules/ and T-cell receptor(s)) involved in β-cell autoimmunity could lead to improved methods of diagnosis as well as therapeutic strategies to prevent IDDM.

In work leading up to the present invention, the inventors sought to identify immunodominant epitopes, in GAD and proinsulin molecules, in order to improve upon current diagnostic procedures and to further develop therapeutic and prophylactic compositions and treatment approaches for IDDM.

In accordance with the present invention, peptides were chemically synthesised based on a thirteen amino acid region of high similarity between the sequences of human GAD 65 (506-518) and human proinsulin (24-36), which region of similarity also extended to human GAD 67 and both mouse proinsulins and GADs (Figure 1). The immunointeractivity of these peptides is identified on the basis of interactivity of peripheral blood cells or T-cells obtained from the peripheral blood of subjects with preclinical or clinical IDDM, thereby forming the basis for a new range of diagnostic, therapeutic and prophylactic procedures for IDDM.

Accordingly, one aspect of the present invention provides recombinant or synthetic peptide molecules of the formula $[X_1]_{n1} - [X_2]_{n2} - [X_3]_{n3}$ wherein:

X1 and X3 may be the same or different and each is a natural or non-naturally occurring amino acid;

X2 is any amino acid sequence derived from, homologous to or contiguous within amino acid sequences of human GAD 65 (506-518) and/or human proinsulin (24-36) including additions, deletions and/or substitutions therein or other derivatives within this region;

n1 and n3 may be the same or different and each is from 0-40;

n2 is not less than about 10 and may be up to about 100; and

wherein said peptides cause T-cell activation when incubated with peripheral blood mononuclear cells (PBMC) or whole anticoagulated peripheral blood obtained from patients having IDDM or who are considered to be at-risk for IDDM.

In a preferred embodiment n2 is not less than about 10 and not greater than about 50, more preferably n2 is not less than about 10 and not greater than about 30 and even more preferably n2 is not less than about 10 and not greater than about 15.

In a particularly preferred embodiment X1, X3, n1 and n3 are as described above and X2 has either of the following amino acid sequences:

F F Y T P K T R R E A E D; or

F W Y I P P S L R T L E D.

The peptides including polypeptides of the present invention may, for example, be prepared by recombinant means. According to this aspect of the present invention, there is provided a recombinant peptide or polypeptide which is preferentially immunologically reactive with Tcells from individuals with clinical or preclinical IDDM, which is prepared by the expression of a host cell transformed with a cassette coding for peptide sequences described above. The peptide or polypeptide may be fused to another peptide or polypeptide. Alternatively, it may be prepared by chemical synthesis, such as by the well-known Merrifield solid-phase synthesis procedure. The synthetic or recombinant peptide or polypeptide may or may not retain GAD activity or proinsulin activity. Furthermore, although synthetic peptides of the formula above represent a preferred embodiment, the present invention also extends to biologically pure preparations of the naturally occurring peptide or peptides or fragments thereof. By "biologically pure" is meant a preparation of at least 60%, preferably at least 70%, more preferably at least 80% and still more preferably at least 90% by weight of peptide.

By "derivatives" is meant to include any single or multiple amino acid substitution, deletion and/or addition relative to the preferred peptide sequence and including any single or multiple substitution, deletion and/or addition to other molecules associated with the peptide or polypeptide including carbohydrate lipid and/or other proteinacious moieties and includes the substitution by any non-naturally occurring amino acids. Such derivatives, therefore, include glycosylated or non-glycosylated forms or molecules with altered glyclosylation patterns.

The invention also extends to use of the peptides and/or polypeptides, or fragments, or derivatives of the present invention in the treatment of patients. In this latter aspect, such

methods of treatment include their use as an adsorbent to remove autoantibodies or autoreactive cells from a patient, their use in direct administration to a patient as a means of desensitising or inducing immunological tolerance or other mechanisms to eliminate or diminish reactivity of autoreactive T-cells or autoantibodies to IDDM autoantigens or to generate T-cell lines or clones to be used for or as therapeutic agents.

As contemplated herein, the method of treatment includes, but is not limited to, the following examples of treatment. A first example of treatment is desensitisation or tolerance induction using an effective amount of synthetic peptide or polypeptide or fragments thereof to alter T-cell recognition of or response to GAD and/or pro-insulin and/or other IDDM antigens and/or induce T-cell suppression. This may be achieved by using the known effect of certain ultraviolet wavelengths, especially UV-B, to modify antigen presentation through the skin (see Ullrich et al (1986) Immunology 58, 158-90). Effective amounts of peptides or polypeptide or fragments thereof would be applied epicutaneously to the skin of subjects exhibiting peripheral blood T-cell reactivity to GAD peptides or polypeptides. After exposure of skin to UV-B radiation, treatment would be repeated until such time that T-cell reactivity to GAD was suppressed. A second example of treatment would be to induce mucosal-mediated tolerance using an effective amount of synthetic peptide or polypeptide or fragments thereof to alter T-cell recognition of or response to GAD and/or pro-insulin and/or other IDDM antigens and/or induce T-cell suppression using an effective amount of synthetic peptide or polypeptide or fragments thereof to alter T-cell recognition of or response to GAD and/or pro-insulin and/or other IDDM antigens and/or induce T-cell suppression by the administration of the synthetic peptide or polypeptide or fragments thereof by oral, aerosol or intranasal means. A third treatment involves application of peptides or polypeptides to the skin together with one or more cytokines such as but not limited to TNFα or β. A fourth treatment involves systemic administration of peptide or polypeptides via subcutaneous or intravenous routes to induce immunological tolerance. A fifth treatment involves T-cell immunisation whereby T-cell lines are generated to GAD peptide or polypeptide or fragments thereof by standard procedures, cells attenuated by fixation with agents such as glutaraldehyde or paraformaldehyde, washed under sterile conditions and re-injected into patients for a time

and under conditions causing suppression of the endogenous T-cell response to autoantigens. These approaches are applicable to the prevention of IDDM progression in asymptomatic subjects with preclinical IDDM or subjects with recent - onset clinical IDDM, as well as to the recurrence of IDDM or in subjects who have received pancreas, islet cell or insulin-producing cell transplants. These approaches are also applicable to Stiff man Syndrome (SMS) and other diseases where GAD and/or proinsulin is an autoantigen. In accordance with the present invention the effective amount of peptide or polypeptide is 0.1 μg to 10 mg per dose and preferably 1.0 μg to 1 mg per dose. A dose may comprise a single administration or an administration protocol. Administration may be by any convenient means such as, but not limited to, intravenous, subcutaneous, epicutaneous, infusion, oral, topical, intranansal, suppository or intraperitoneal administration. The peptide or polypeptide may be administered alone or in combination with one or more other active molecules, molecules which facilitate the peptide or polypeptide activity such as, LPS, choleratoxin β-chain LFA-3, other adjuvants and in particular, TNF-α and/or TNF-β.

In yet a further embodiment, the present invention contemplates the use of the synthetic peptide or polypeptide to measure reactivity of a subject's cells to the IDDM autoantigen. The peptide or polypeptide, or fragments or derivatives thereof, may be added, in solution or bound to a solid support together with cells from a subject derived from peripheral blood or from tissue biopsies either unfractionated, fractionated or derived as continuous cell lines. Reactivity to the autoantigen may then be measured by standard proliferation assays such as incorporation of tritiated thymidine, standard cytotoxic assays such as release of marker radioactivity from target cells, measurements of expressed or secreted molecules such as cytokines or other standard assays of cellular reactivity which are well known in the art.

In one embodiment of this aspect of this invention there is provided a diagnostic kit for assaying T-cells. Standard 96 - well plates, as used in ELISAS, are pre-coated with a monoclonal antibody (MAb) to a T-cell cytokine such as γ-interferon (γ-IFN) with or without antigen. Alternatively, antigen is added in soluble form together with aliquots of

peripheral blood, peripheral blood mononuclear cells or Tcells. Incubation is allowed to proceed for one or more days, the supernatant (medium, plasma) and the cells are washed off, wells washed again and plates developed with a labelled second MAb to the cytokine such as anti-γ-IFN conjugated with alkaline phosphatase or horseradish peroxidase. Colorimetric reaction and read-out systems can then be utilised. Alternatively, soluble cytokines (eg: γ-IFN) are measured in the supernatant by standard assays such as ELISA; further it is possible to visualise microscopically by the ESLIPOT technique individual spots on bottoms of wells representing cytokine produced at the single cell level, thereby enabling the precursor frequency of antigen-reactive T-cells to be determined.

The present invention will now be further described with reference to the following non-limiting Figures and Examples.

In the Figures:

Figure 1 shows a comparison of the regions of similarity among mouse and human proinsulins and GADs. Similarities are boxed; identities within boxes are shaded. The C-terminus of the mature insulin B-chain and the pro-insulin cleavage site are indicated by the vertical line and arrow respectively.

Figure 2 is a graphical representation showing the level of cellular proliferation expressed as the delta score following the stimulation of perpheral blood mononuclear cells taken from IDDM at-risk or control subjects with the following peptides: human GAD65 (506-518); human proinsulin (24-36); irrelevant control peptide; or tetanus toxoid (CSL Ltd).

EXAMPLE 1
Materials and Methods

***Subjects*:**

Subjects at-risk for IDDM were from the Melbourne Prediabetes Family Study. Each was entered on the basis of having at least one first degree relative with IDDM, and islet cell antibodies (ICA)≥20 JDF units and/or insulin autoantibodies (IAA)≥100nU/ml. All had normal fasting blood glucose and glycated hemoglobin and have had repeat antibody and metabolic tests at six monthly intervals.

Control subjects were HLA-DR matched, asymptomatic, and without history of IDDM.

All subjects gave informed, signed consent and the study was approved by the Ethics Committees of the Royal Melbourne Hospital and the Walter and Eliza Hall Institute of Medical Research. Details of Subjects are described in Table 1.

Table 1

Subject #	Age	Years Follow-up	HLA				ICA *	IAA †	GAD Ab £	FPIR ¶
			A	B	DR	DQ				
1	14	1.6	1	8	3	2	160,69,56	4,30,-20		118,155
2	23	4.8	2	44,55	3,4	5,8	55,37,14,6,5,5	-25,9,41,-2,0,44	278,602	124,113,57
3	22	6.8	2,28	7,8	3,4	2,8	37,37,37,37,52,30,58,46,26	8,9,2,31,7,9,-41,-1,64	1637,2259,634,1535	183,155,140,161,56
4	13	1.3	1,11	8,27	3,4	2,8	160,190	84.280		79,91
5	25	5.5	2	44,62	4,11	7,8	0,19,18,16,22,0,0	45,31,42,60,29,130,30	736,936,1336,790,810	137,143,68,15
6	20	5.5	1,2	8,62	3,4	2,8	19.19.104.86.8	480,560,400,130,300	937,2258,2389	105,238,165,128
7	18	1.7	1,3	8,18	3	2	69,69	13,20		44,47
8	9	3.2	1,2	8,44	3,4	2,8	160,160,160,160	-2,-26,36,59	2300,1830	118,129,87
9	10	2.8	1,2	8,27	3,4	2,8	160,160,120,24	2,29,14,120	1525,1388	26,56,29
10	14	4.8	1,32	8,14	4,7	2,8	14,13,51,18	240,490,470,1000	432	318,181,165

* ICA=islet cell antibody titres (JDF units)

† IAA=insulin autoantibody titres (nU insulin bound/ml serum)

£ GADAb=glutamic acid decarboxylase autoantibody titres (nU/ml)

¶ FPIR=first phase insulin release (sum of serum insulin concentrations at 1 and 3 minutes following completion of glucose injection)

HLA typing and assays of ICA, IAA, GAD Ab, FPIR:

HLA Typing:

HLA class I (A, B, C) and HLA class II (DR,DQ) typing was performed using populations of T and B lymphocytes respectively. The cells were isolated from anticoagulated blood using magnetic beads coated with monoclonal antibodies to CD8 (class I) or a monomorphic determinant on the class II beta chain (class II) (Dynal). The enriched cell populations were typed in a standard microlymphocytotoxicity assay using a battery of 240 allosera for class I and 120 allosera for class II.

Antibody assays:

ICA were assayed using indirect immunofluorescence on blood group O donor pancreas. Titres, in JDF units, were determined by doubling dilution of positive sera and comparison with standard sera run in each assay. The assay has been included in all International Diabetes Workshops and proficiency programs.

IAA were assayed by a radiobinding assay which has been internationally standardised. The the upper limit for normal control sera is 40 nU insulin bound/ml serum.

GAD antibodies were assayed by immunoprecipitation of GAD enzymatic activity from piglet brain extract . The mean plus 3 SD of 72 healthy subjects, 460nU/ml, was used to define the normal range.

First phase insulin release (FPIR):

FPIR was calculated as the sum of serum insulin concentrations at 1 and 3 minutes following the completion of intravenous glucose (0.5g/kg body weight) injected over 3 minutes.

T cell proliferation assay:

Blood was drawn from paired IDDM at-risk and HLA-DR matched controls at the same time (within 30 minutes) and processed similarly to reduce the effects of diurnal variation and handling artefacts. Peripheral blood mononuclear cells were isolated from heparinised whole blood by Ficoll-Paque (Pharmacia Biotech) density centrifugation, washed and resuspended in RPMI 1640 medium (Biosciences Pty Ltd) containing 20mM Hepes (CSL Ltd), 10^{-5} M 2-mercaptoethanol (BDH), penicillin (100U/ml), streptomycin (100µg/ml) and 10% autologous plasma. Aliquots of 200µl ($2x10^5$ cells) were transferred into wells of a 96-well, round-bottomed plate (Falcon) and incubated in replicates of six with the following peptides at final concentrations of 10, 2, and 0.4µg/ml: human GAD65 (506-518), human proinsulin (24-36) (synthesised using an Applied Biosystems Model 431A synthesiser (ABI, Foster city, CA), and an irrelevant control peptide (CRFDPQFALTNIAVRK) (Macromolecular Resources, Fort Collins, CO). Tetanus toxoid (CSL Ltd) at final concentrations of 1.8, 0.18 and 0.018 LfU/ml was used as a positive control. Twelve "autologous only" wells containing cells but without antigen were included as the background control. Plates were incubated at 37°C in a 5% CO_2 humidified incubator for 6 days; 0.25µCi of [^{3}H]thymidine (ICN) was added to each well for the last 6 hours. The cells were than harvested onto glass fibre filters and incorporated radioactivity measured by beta-particle counting (Packard Model 2000 Liquid Scintillation Counter). The level of cellular proliferation was expressed as the delta score (DS=mean counts per minute (cpm) incorporated in the presence of antigen, minus the mean cpm of the "autologous only" wells).

EXAMPLE 2

Results

T-cell proliferative responses to the similar 13-mer peptides from proinsulin and GAD were compared for ten pairs of HLA-DR matched at-risk and control subjects. HLA-DR matching was thought to be important not only because of the specificity of peptide binding to MHC class II alleles but also because of the known association between MHC class II and IDDM. Therefore T-cell responses would reflect IDDM rather than MHC specificity . Responses to the highest concentration of either peptide were significantly (proinsulin, $p<0.008$; GAD, $p<0.018$ - Wilcoxon one-tailed paired analysis) greater among at-risk than control subjects. The results are summarised in Table 2.

Table 2

Pair #		Delta Scores*													
		Autologous		Proinsulin 10 µg/ml		Proinsulin 2 µg/ml		Proinsulin 0.4 µg/ml		GAD 10 µg/ml		GAD 2 µg/ml		GAD 0.4 µg/ml	
		At Risk	*Control*	*At Risk*	*Control*	*At Risk*	*Control*	*At Risk*	*Control*	*At Risk*	*Control*	*At Risk*	*Control*	*At Risk*	*Cont*
1		881	2979	1391	0	459	0	1040	0	579	0	516	0	768	0
2		236	389	351	0	0	0	33	0	3263	0	190	0	199	0
3		6515	217	0	64	355	43	0	0	0	77	0	5	0	25
4		595	1347	104	0	0	0	288	0	0	0	10	0	0	0
5		1745	1269	694	0	0	0	0	0	1275	20	394	120	53	30
6		1007	265	397	98	65	380	0	0	1679	992	220	216	77	195
7		1392	454	467	93	0	0	0	0	2313	1365	0	0	0	70
8		9993	308	2128	0	1367	0	0	0	0	0	0	0	0	0
9		598	135	0	0	265	13	0	0	1251	337	0	0	255	21
10		597	870	56	21	0	22	0	0	65	391	0	1441	0	0
	Mean	2355.8	823.4	558.7	27.6	251.1	45.8	136.0	0	1042.4	318.1	133.0	178.2	135.3	34.1
	Std. Error	1025.7	276.2	219.5	13.0	135.3	37.4	104.4	0	357.1	153.0	60.5	142.2	76.0	19.2
	Wilcoxon P-Value (One-Tailed)			0.008		0.125		0.054		0.018		0.199		0.199	

* Delta Score=mean of six replicate wells minus mean of twelve autologous wells (if less than 0, shown as 0)

Reactivity to the proinsulin sequence was confined almost entirely to at-risk subjects, whereas some controls also responded to the GAD peptide (Table 2, Fig. 2). Both groups responded similarly to tetanus, and no subject reacted to the unrelated control peptide.

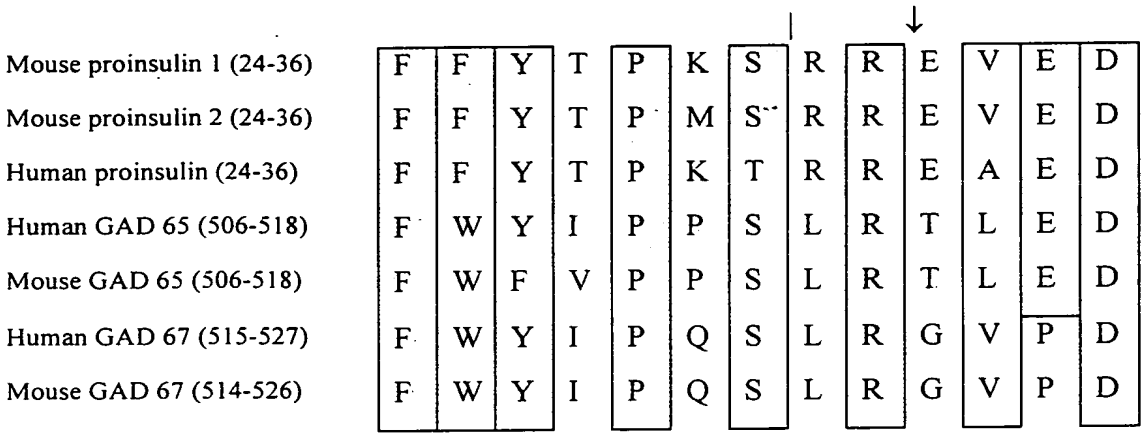
For six of these pairs (#1, 2, 3, 5, 6, 7) the assay was performed on a separate occasion, but using twice as many cells ($4x10^5$ per well). Exhaustion of the media resulted in unreliable results in three cases. In two of the other three (#5 and 6), the results were consistent with those tabulated here, while in the third (#3) the at-risk subject displayed greater reactivity to both antigens at the higher cell number.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

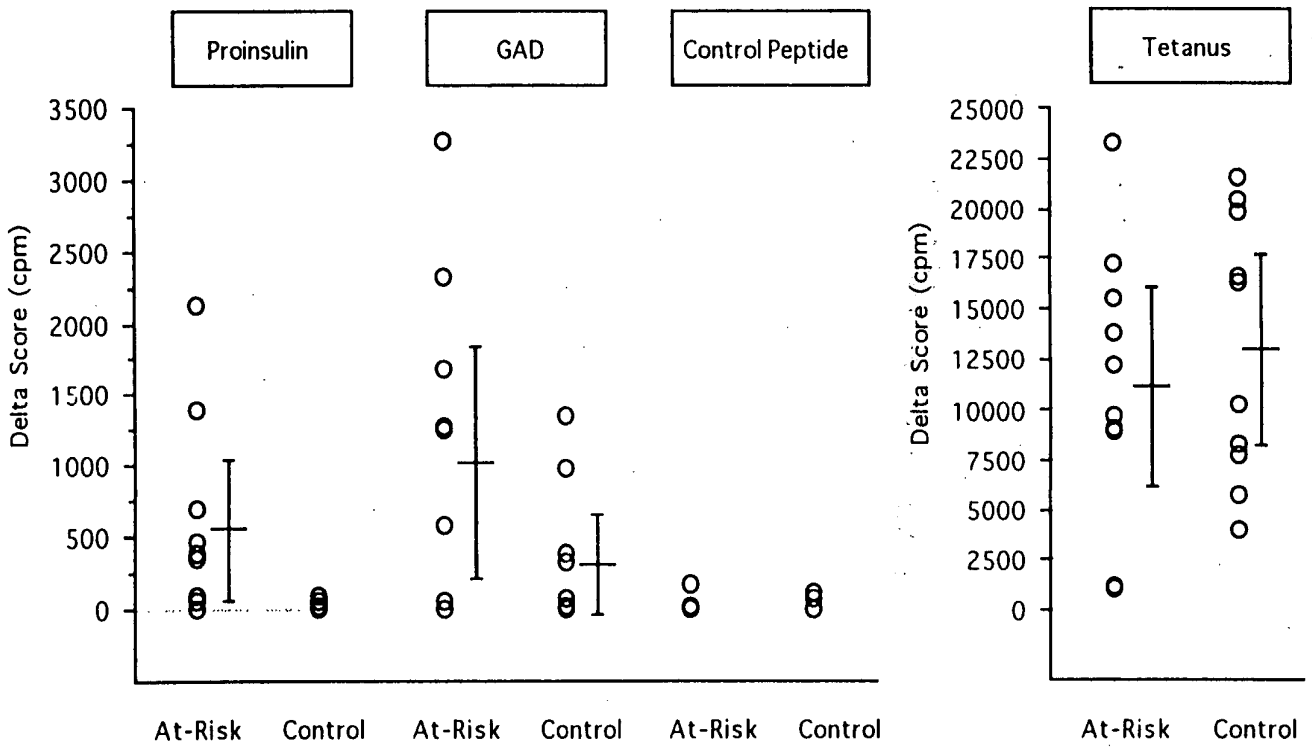
DATED this 4th day of September, 1995

THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH
By Its Patent Attorneys
DAVIES COLLISON CAVE

Figure 1



Region of similarity among mouse and human proinsulins and GADs. Similarities are boxed; identities within boxes are shaded. The C-terminus of the mature insulin B-chain and the pro-insulin cleavage site are indicated by the vertical line and arrow, respectively.


Proinsulin
GAD
Control Peptide
Tetanus
Delta Score (cpm)
3500
3000
2500
2000
1500
1000
500
0
25000
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
At-Risk
Control
At-Risk
Control
At-Risk
Control
At-Risk
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